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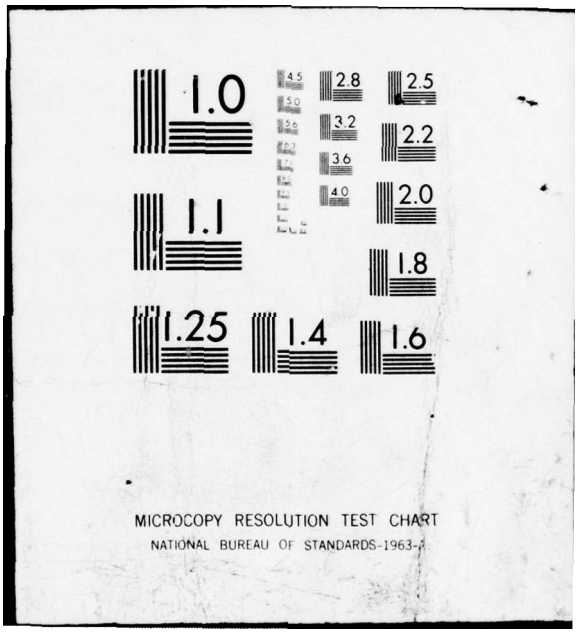
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LAIR REPORT NO.41

COMMON BLOOD BANK CONTAMINANTS EVALUATED BY THE BACTEC RADIOMETRIC SYSTEM

BLOOD RESEARCH DIVISION
DEPARTMENT OF SURGERY

AUGUST 1977

LETTERMAN ARMY INSTITUTE OF RESEARCH PRESIDIO OF SAN FRANCISCO CALIFORNIA 94129

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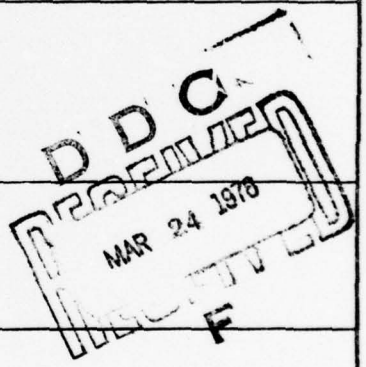

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Research techniques in blood preservation require frequent entry into blood bags stored at 4°C. Rapid detection of possible contamination and careful evaluation of a selected bacteriological method are essential. Bactec® radiometric methods (Johnston Laboratories, Inc., Cockeysville, MD) were evaluated. Data were collected concerning detection of small numbers of organisms (1 to 10^3 colony forming units per milliliter). Studies were done with 0.5 to 3 ml/ vial inoculum and at temperatures of 35,22-24,and 4°C. The evidence indicates that the Bactec® radiometric procedure is adaptable to

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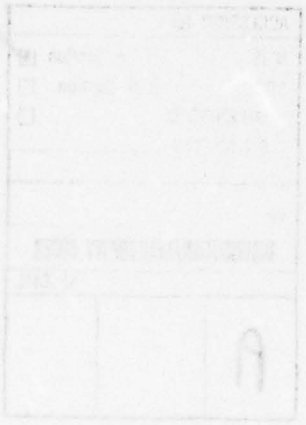
smaller samples and to different incubation temperatures than recommended by Bactec[R] for non-fastidious organisms only. Oxidative psychrophilic and other fastidious organisms were detected more frequently and faster using subculture and gram stain methods.

ABSTRACT

Research techniques in blood preservation require frequent entry into blood bags stored at 4°C. Rapid detection of possible contamination and careful evaluation of a selected bacteriological method are essential. Bactec® radiometric methods (Johnston Laboratories, Inc., Cockeysville, MD) were evaluated. Data were collected concerning detection of small numbers of organisms (1 to 10^3 colony forming units per milliliter). Studies were done with 0.5 to 3 ml/vial inoculum and at temperatures of 35, 22-24, and 4°C. The evidence indicates that the Bactec® radiometric procedure is adaptable to smaller samples and to different incubation temperatures than recommended by Bactec® for non-fastidious organisms only. Oxidative psychrophilic and other fastidious organisms were detected more frequently and faster using subculture and gram stain methods.

PREFACE

The authors are indebted to Lottie Applewhite and Celeste Mangold for their many helpful suggestions and technical assistance.



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INTRODUCTION

Microbiological techniques for blood banks must include the use of incubation temperatures that will permit growth of cold-growing (psychrophilic) organisms (1). Although many psychrophilic organisms will grow over a wide temperature range, some will not be detected at 37 C and most have an optimum growth temperature of about 20 C (2,3). Because many of the psychrophils implicated in blood bank contamination are members of the oxidative gram negative rod group of bacteria, they may produce toxins that pose a threat to the potential transfusion recipient. Braude et al (4,5) demonstrated the rapid multiplication of these bacteria in blood stored at 4 C and also reported that 25% of warm-growing (mesophilic) contaminants (which grow better at 35 C) will also multiply in 4 C blood when originally present at 1 to 10^3 colony forming units per milliliter (cfu/ml). Braude et al (4,5) further stated that the number of organisms encountered in the initial contamination of blood usually fell within the range of 1 to 10^3 cfu/ml.

Blood preservation research techniques require frequent entry into blood bags stored at 4 C, a procedure which greatly increases the risk of bacterial contamination. Therefore, rapid detection of possible contamination in these circumstances is essential, and careful evaluation of the selected bacteriological method is mandatory. Although radiometric detection of bacterial growth has been reported (3, 6-9) most of these studies involved 37 C incubation of blood cultures from clinically ill patients. Myhre et al (3) have sought, however, to study frozen-reconstituted blood units from the blood banks and to include in their final culturing protocol an aerobic vial cultured at 20 C. They evaluated the Bactec® instrument as a rapid method for determining the sterility of the blood.

The purpose of our investigations was to evaluate the ability of the Bactec® instrument to detect common blook bank contaminants, and, in addition, to determine whether or not Bactec® radiometric methods would be applicable to testing smaller than recommended samples and adaptable

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1. American Association of Blood Banks. Technical Methods and Procedures (Sixth edition). 1974. p 215
2. Ingraham, J. L. In: The Bacteria, I.C. Gunsalus and R.Y. Stanier (eds). 1962. pp 265-296
3. Myhre, B.A. et al. Transfusion 17:195, 1977
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to different incubation temperatures (10). Data were collected concerning detection of small numbers of organisms (1 to 10^3 cfu/ml) usually involved in initial blood contamination. Studies were done with less than the 3 ml/vial inoculum recommended by Bactec^R procedures and samples were incubated at room temperature (22 to 24 C) and 4 C for psychrophilic contaminants. The evidence indicates that the Bactec^R radiometric procedure is adaptable to smaller samples and to incubation temperatures different from those recommended by the manufacturer of Bactec^R, however, it presents no advantage and some disadvantages in the detection of psychrophilic and other fastidious organisms.

MATERIALS AND METHODS

MATERIALS

This study was performed with the Bactec^R R301 procured from Johnston Laboratories, Inc. (3 Industry Lane, Cockeysville, MD 21030) (10). The Bactec^R procedure is summarized as follows. If a microorganism is inoculated into the Bactec^R vial, the organisms metabolize the ^{14}C substrates which are in the vial; thus, $^{14}CO_2$ is liberated into the atmosphere of the vial. The instrument analyzes the gas for radioactivity. When a threshold level is exceeded by a reading over a designated index number (display number), the vial is positive, i.e., the sample inoculum contains viable organisms.

Media. Culture vials containing ^{14}C-labelled substrates were obtained from Johnston Laboratories, Inc. Bactec^R aerobic 6A vials were used on trials of all the microorganisms. In addition, Bactec^R 3A (hypertonic vials) were obtained and used for Enterococcus trials. The Bactec^R R301 was routinely checked with the performance blood culture kit controls. Brain heart infusion broth (BHI), trypticase soy agar (TSA) tubes, and blood agar plates (BAP) were obtained from Baltimore Biological Laboratories (Box 243, Cockeysville, MD 21030). Two tubes or plates from each lot were incubated (one at 22-24 C, another at 35 C) for 24 hours to insure sterility. During the same 24 hours, a tube or plate from each of the same lots was inoculated with stock organisms to evaluate its ability to support growth. Blood specimens, obtained from healthy volunteers, were drawn into citrate-phosphate-dextrose (CPD) solution. A portion of each specimen was cultured aerobically and anaerobically to verify sterility; the blood was used as medium for the Bactec^R experiment.

Microorganisms. Stock cultures (Table 1) were obtained from Department of Pathology (Reference Microbiology Laboratory), Letterman Army Medical Center, Presidio of San Francisco, California, and from Letterman Army Institute of Research. The cultures were stored frozen on alundum beads (Cat. No. A620, Fisher Scientific Co., 60 mesh "RR"). Aliquots were

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10. Johnston Laboratories, Inc. BACTEC Data. JLI-513B. Nov 20, 1975

subcultured to BHI and used within 48 hours. Cultures were regularly subcultured from the alundum beads (11), checked for purity by streaking for isolated colonies, and screened for proper biochemical reactions with API 20E (Analytab Products, Inc., 200 Express Street, Plainview, NY 11803) and Oxi/ferm^{tm} (Roche Diagnostics Division of Hoffman-LaRoche, Inc., Nutley, NJ 07110) identification systems. Oxidative organisms were sent to the Department of Pathology (Reference Microbiology Laboratory), Letterman Army Medical Center, for species confirmation.

Instrumentation. An Eberback #6130 rotating shaker and New Brunswick metabolite water bath shakers, Models G77 and G76, were used for agitation of the Bactec^{R} vials. Incubation was achieved at 35 C in a Forma Model 3151 CO_2 incubator, at room temperature (22 to 24 C) within the laboratory and a 4 C in a temperature-monitored refrigerator.

METHODS

BHI subcultures of the stock organisms were plated onto blood agar to obtain isolated colonies. By using one colony of each organism, serial dilutions with subsequent pour plates and colony counts were prepared to establish the proper dilution needed to obtain 1 to 10^3 cfu/ml (12). Selected organisms were mixed with the blood to simulate contaminated blood bag samples. All organism dilutions, both in BHI and blood, were inoculated, incubated, and read according to the format outlined in Figure 1. The 35 C, room temperature, and 4 C vials were shaken for the first 24 hours at speeds determined by Johnston Laboratories. Depending upon the instrument used, this criterion was met with speeds between 240 and 265 rpm. Positive results were defined as a Bactec^{R} growth index (instrumentation display number) of 20 or higher for BHI inoculums and 30 or above for blood inoculums.

RESULTS

Bectec^{R} reading times in Figure 1 varied slightly with the organism, its expected growth rate (13), and the incubation temperature. As can be seen in Table 2, within 72 hours the cold-growing organism (incubated at 22 to 24 C) and the warm-growing organisms (incubated at 35 C) were Bactec^{R} positive with the exception of the Enterococcus which did not grow in the 6A blood inoculated vials. Furthermore, 12 of the 16 microorganisms were Bactec^{R} positive in less than 24 hours; the others, P. fluorescens, P. cepacia, and P. maltophilia had a range between 24 and 72 hours. In the organisms with less than 24-hour

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11. California State Department of Public Health. Method for Storing Stock Cultures (alundum technique). (no date)
12. Becton, Dickinson and Company. BBL Manual of Products and Laboratory Procedures (Fifth edition). 1973. pp 4-5
13. Waters, J.R. Appl Microbiol 23:1988, 1972

detection times (with the exception of three BHI trials which had colonies too numerous to count), the colony counts ranged from 15 to 470 cfu/ml in both the BHI and blood inoculum. Between 24 and 72 hours, the colony counts ranged from 2 to 96 cfu/ml for blood inoculum and from 4 to 224 cfu/ml in the BHI inoculum. Neither the kind of inoculum (blood or BHI) nor the quantity of inoculum (3 ml, 1 ml, or 0.5 ml) produced a noticeable difference in detection time for most of the organisms.

All the psychrophilic cultures of Pseudomonas fluorescens were Bactec^R positive at room temperature before they became positive at 35 and 4 C incubation. Average detection times for P. fluorescens at the three different temperatures were as follows: 45 hours at 22 to 24 C, 128 hours with one negative trial at 35 C; 293 hours with one negative trial at 4 C.

Table 3 contains results of our visual inspection of the clear Bactec^R vials after they were inoculated with clear BHI-diluted organisms. With different organisms, we observed a 2-to-47-hour lag time between first visual cloudiness and first positive Bactec^R growth index. Eight subcultures to blood plates and additional colony counts were prepared from cloudy but Bactec^R negative vials and resulted in good growth with organisms too numerous to count. None of the eight vials, consisting of five P. fluorescens cultures and three Enterococcus cultures, ever became Bactec^R positive and should be classified as false negatives. Two additional subcultures and colony counts were performed on Enterococcus vials that had become Bactec^R negative by 24 hours. Both subcultures produced colonies too numerous to count. In no case did a vial read positive on the Bactec^R instrument unless it was visually cloudy; conversely, no visually clear vial ever produced a positive Bactec^R growth index. It should be noted, however, that the visual cloudiness can only be observed when clear inoculum is utilized; i.e., when blood was inoculated the cloudiness could not be observed.

DISCUSSION

Preliminary studies performed in our laboratory, in which seeded blood and Bactec^R detection times for contaminated stored blood were compared, indicated no significant differences. Using seeded blood and the Bactec^R system, we were able to detect most of the common blood bank contaminants within the range of 1 to 10^3 cfu/ml before 72 hours. Medium and amount of inoculum made no practical difference in the detection times for any organism except the Enterococcus. The long lag times and the fact that several organisms grew well in the vials but did not produce positive growth indices indicate the necessity for early subculture when fastidious organisms are suspected. Some of the organisms (Enterococcus and P. fluorescens)would have been judged negative unless subcultured or gram stained; some organisms (P. maltophilia and P. cepacia) could have been detected considerably earlier if they had been subcultured at 24 hours.

Room temperature incubation was not only applicable to Bactec^R usage but also necessary for the detection of the psychrophilic P. fluorescens. This species has frequently been implicated in blood bag contamination (4, 14). All P. fluorescens cultures were first detected at room temperature and sometimes they were not detected at 4 or 35 C. It is apparent that room temperature incubation produced more consistent detection faster than any other temperature we used. The Pseudomonas organisms had slower detection rates, longer lag times, and lower CO_2 production, which are reflections of the growth characteristics of the oxidative gram-negative rod group of organisms. Many of these organisms utilize glucose with or without gas production slowly or not at all (15). Our P. maltophilia did not utilize glucose at all; however, its positive (but low) Bactec^R growth index indicated conversion of one of the other Bactec^R ^{14}C-labelled substrates to $^{14}CO_2$.

The results obtained with the Enterococcus trials represent only one culture strain and thus must be considered with caution. However, in no instance did we observe a Bactec^R reading of greater than 40. When Enterococcus was incubated in blood inoculum, positive results were obtained with the 8A hypertonic vials but not with the 6A vials. It is possible that bacteriostatic properties of blood interfaced with our ability to detect Enterococcus, but we did not observe that phenomenon with any other organism. Although Bactec^R 8A positive growth indices were obtained on some of the vials before the 24-hour reading (i.e., ±16 hours), these decreased to negative readings by 24 hours. We propose that Enterococci Bactec^R 6A and 8A vials be subcultured at 24 hours even if the growth indices are negative. Samples should be subcultured onto appropriate medium (6).

CONCLUSIONS AND RECOMMENDATIONS

Although the Bactec^R radiometric procedure proved adaptable to smaller samples than recommended by the manufacturer of Bactec^R and to different incubation temperatures necessary for the detection of 1 to 10^3 cfu/ml of common blood bank contaminants, it presented no real advantage and some disadvantages in the detection of certain organisms. When Bactec^R procedures are used to detect oxidative, psychrophilic, and other fastidious organisms encountered in blood contamination, early subculture and gram stain should be performed to eliminate false negatives.

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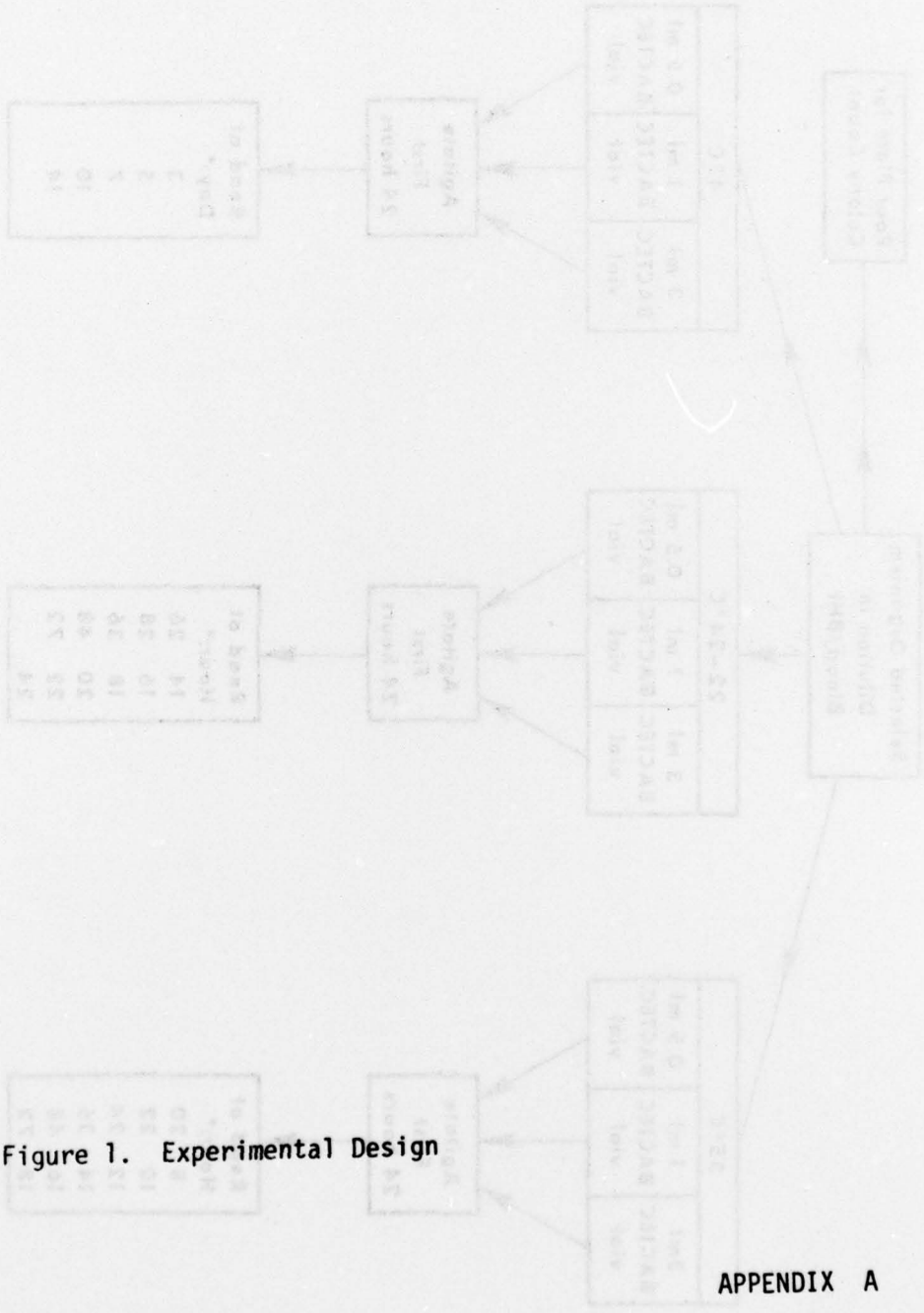
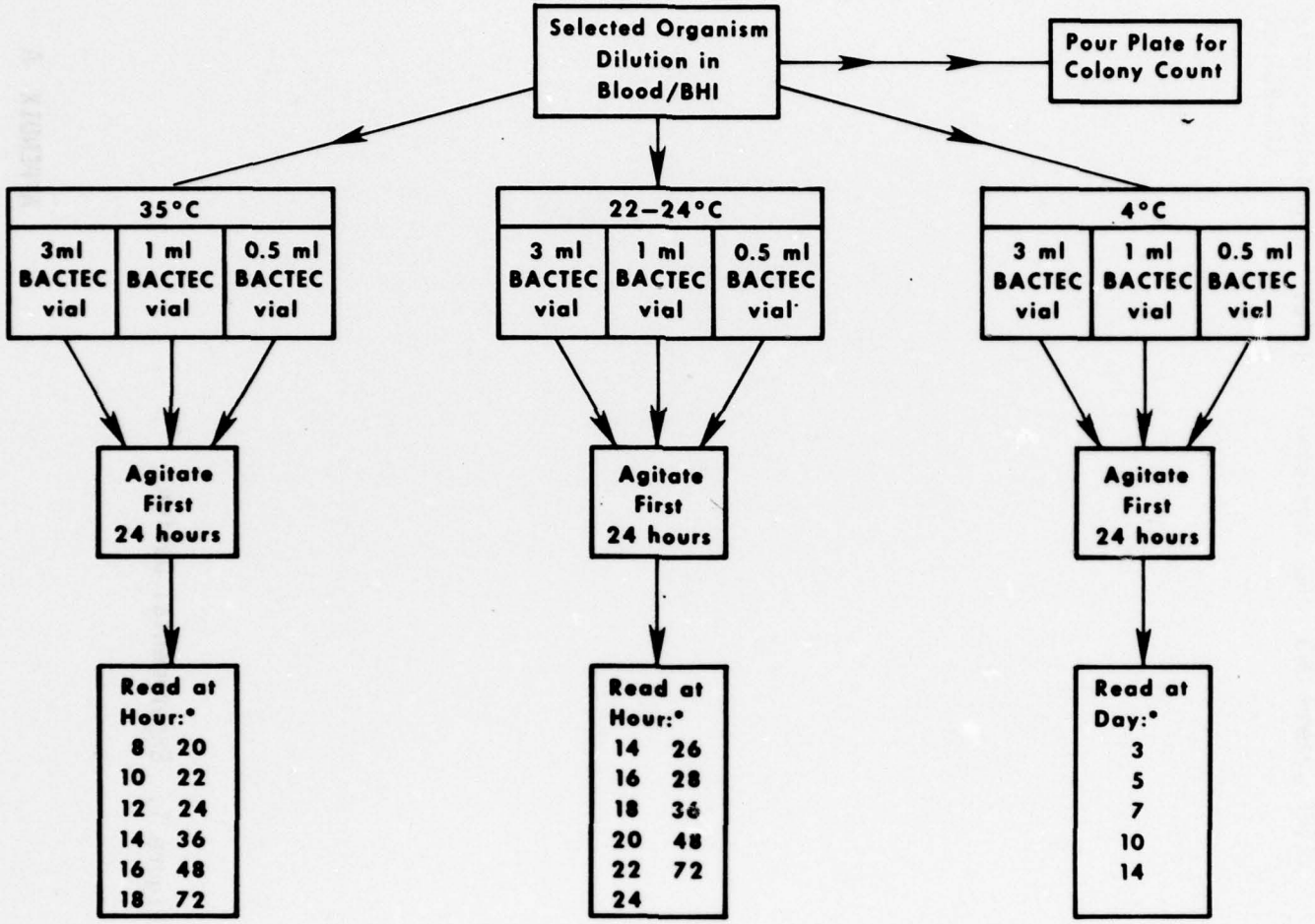


Figure 1. Experimental Design

APPENDIX A

FIGURE 1. EXPERIMENTAL DESIGN



* subculture or gram stain performed on low growth index and occasional cloudy vial

APPENDIX B

TABLE 1

MICROORGANISMS TESTED

Organism	Number of Trials
Escherichia coli	3
Citrobacter freundii	3
Klebsiella pneumoniae	1
Enterobacter cloacae	1
Enterobacter aerogenes	1
Yersinia enterocolitica	1
Serratia liquefaciens	1
Serratia marcescens	1
Vibrio alginolyticus	1
Pseudomonas aeruginosa	2
Pseudomonas fluorescens	3
Pseudomonas maltophilia	3
Pseudomonas cepacia	2
Staphylococcus aureus	2
Staphylococcus epidermidis	2
Enterococcus	3

TABLE 2: TIME REQUIRED FOR POSITIVE BACTEC INDEX WITH ORGANISMS CULTURED AT 35°C (except *P. fluorescens* cultured at 22-24 C)

Organism	Inoculum Colony Count per Trial (cfu/ml)	Hours to Detection in BHI Inoculum			Hours to Detection in Blood Inoculum		
		3 ml	1 ml	0.5 ml	3 ml	1 ml	0.5 ml
E. coli	TNTC[†]	10	10	10	...	...	...
	185	...	...	...	10	10	10
	65	12	12	12	...	...	...
C. freundii	220	12	12	12	...	...	...
	130	...	...	...	12	12	12
	25	10	...	10	...	...	...
	15	12	12	12	12	12	12
K. pneumoniae	280	...	...	...	10	10	10
	250	10	10	10	...	...	...
E. cloacae	n.r.**	12	12	12	...	...	...
	58	...	...	...	12	12	12
E. aerogenes	108	12	12	12	...	...	...
	5	...	...	...	12	12	12
Y. enteroco-	308	16	16	16	...	...	...
litica	260	...	...	...	16	16	16
S. liquefaciens	470	12	12	12	12	12	12
S. marcescens	345	...	...	...	10	10	10
V. algino-	107	10	10	10	...	...	...
lyticus	33	...	...	...	10	10	10
P. aeruginosa	TNTC	18	...	20	...	...	...
	45	...	...	...	19	19	19
	37	19	19	19	...	...	...
P. fluorescens	224	44	44	44	...	...	...
22-24 C	70	48	...	48	...	...	...
	60	...	...	...	44	44	44
	34	44	44	44	...	...	...
	9	...	...	...	44	72	72

TABLE 2, continued

Organism	Inoculum Colony Count per Trial (cfu/ml)	Hours to Detection in BHI Inoculum 3 ml	1 ml	0.5 ml	Hours to Dectection in Blood Inoculum 3 ml	1 ml	0.5 ml
P. maltophilia	17	...	...	...	44	44	44
	8	44	44	n.d.*	...	...	...
	4	65	...	65	...	...	...
P. cepacia	96	...	...	...	24	24	24
	64	...	22	...	...	...	...
	10	30	26	30¶	...	...	...
	2	...	...	...	30¶	30¶	26
S. aureus	130	...	...	...	24	13	13
	53	12	12	12	...	...	...
S. epidermidis	162	...	16	...	...	...	...
	142	...	...	...	48	20	20
	70	16	16	16	...	...	...
Enterococcus††	16	22	...	...	...	...	...
	<10	...	...	...	n.d.	n.d.	n.d.
	3	24	...	12	...	...	...
8A	8	...	...	...	16	16	n.d.
	3	14	...	n.d.	...	...	...

†too numerous to count

*never became Bactec^{R} positive

**not readable

¶was detected, but within range of 30-47 h. Thirty hours almost positive; next reading at 47 hours showed well over positive threshold.

††Bactec^{R} positive on the hours stated; however, became negative by 24 hours. Twenty-four hour colony count was too numerous to count.

TABLE 3. COMPARISON OF DETECTION TIMES FOR GROWTH

Organism		First Cloudiness Observed (hours)	First BACTEC Positive (hours)	BACTEC Lag Time (hours)
E. coli		8	10	2
C. freundii		8	12	4
K. pneumoniae		8	10	2
Y. enterocolitica		13	16	3
S. liquefaciens		8	10	2
S. marcescens		8	10	2
V. alginolyticus		8	10	2
P. aeruginosa		14	18	4
P. fluorescens	35°C	38	65	27
	22-24°C	26	48	22
		27	44	17
		24	44	20
	4°C	~192±24	240	~47±24
		192	336	144
		312	n. det.*	
P. maltophilia		18	65	47
		24	44	20
S. aureus		10	13	3
S. epidermidis		12	16	4
Enterococcus	6A BHI	10	24	14
	6A BHI	12	22	10
	6A Blood	n.d.*	n.d.*	n.d.*
	8A BHI	10	14	4
	8A Blood	13**	16**	3**

* not detected

** Became BACTEC negative by 24h. Twenty-four h colony count was too numerous to count.

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